

Amendments to the Specification

Please replace the paragraph at page 1, lines 6-9, with the following amended paragraph:

This application is a divisional of ~~co~~pending U.S. Application No. 09/264,747, filed March 09, 1999, now U.S. Patent 6,645,503, the entire disclosure of which is hereby incorporated by reference, which claims ~~the benefit of~~ priority to U.S. Provisional Application No. 60/088,364 which was converted from U.S. Patent Application No. 09/037,529, now abandoned, filed March 10, 1998, pursuant to a petition filed under 37 C.F.R. 1.53(c)(2) filed May 6, 1998.

Please replace the paragraph at page 13, lines 9-21, with the following amended paragraph:

Vaccines containing the antigenic conjugates of the present invention may advantageously contain various adjuvants which are known to augment the immune response to the vaccine antigen. It is believed that such adjuvants increase the antibody response by the non-specific stimulation of the patient's immune system. The use of adjuvants is well known in the art and is described, e.g., in "Vaccine Design: The Subunit and Adjuvant Approach", Powell et al., Plenum Press (1995). Examples of adjuvants suitable for use in vaccines containing the present conjugates include: aluminum phosphate, aluminum hydroxide, monophosphoryl lipid A, 3-deacylated monophosphoryl lipid A, ~~QS-21 (as disclosed in J. Immunol., 146:431-437 (1991))~~ STIMULON™ QS-21 (Antigenics, Framingham, Massachusetts, as disclosed in J. Immunol., 146:431-437 (1991)); as well as various detergents (e.g., Triton™X100, zwittergents and deoxycholate) in combination with the aluminum compounds. In general, the antibody response to the present conjugates is substantially increased by the inclusion of one or more adjuvants in the vaccine.

Please replace the paragraph at page 16, lines 13-21, with the following amended paragraph:

The LPS purified in the manner described above, was then de-O-acylated by the reaction thereof with 45 mM of NaOH at 80°C for 20 minutes. The de-O-acylated material was then neutralized with HCl and purified by gel filtration on a ~~BioGel P6 column~~ Bio-Gel® P6 column (Bio-Rad® Laboratories, Inc.; Hercules, CA) using 0.1 M NaHCO₃ as an eluant. The de-O-acylated LPS (referred to hereinafter as “DeA-LPS”) was then conjugated to the carrier protein CRM₁₉₇ by linking the amino groups of the saccharides on the conserved LPS structure to the amino groups of the carrier protein utilizing the procedure described below. CRM₁₉₇ is a non toxic mutant protein of diphtheria toxin and has been used as a carrier protein for the commercial production of glycoconjugate vaccines for human use.

Please replace the paragraph at page 16, line 23 through page 17, line 5, with the following amended paragraph:

Long chain sulfo-N-succinimidyl-3-(2-pyridyldithio)-propionate (sulfo LC-SPDP available from the Pierce Chemical Company) was used to thiolate the primary amino group(s) of the DeA-LPS. The sulfo LC-SPDP was added to 15 mg of the LPS in 0.1 M NaHCO₃ (pH 7.9) at a ratio of 1:1 (w/w). This mixture was then incubated for an hour at room temperature. At the end of the reaction, the mixture was purified on a ~~BioGel~~ Bio-Gel® P6 column equilibrated in 0.1 M NaHCO₃. The recovered fractions were assayed for KDO according to the procedure set forth in Keleti and Lederer, Biochem. Biophys., 74:443-450 (1974) and the fractions containing the KDO were pooled. The N-pyridyl disulfides present in the SPDP derivatives of the LPS were reduced with 50-100 mM dithiothreitol (DTT) and gel filtered on a ~~BioGel~~ Bio-Gel® P6 column as described above. The thiolated material containing the KDO positive fractions were again pooled. Thiolation of the oligosaccharides

of the DeA-LPS was monitored in accordance with the reaction described in Ellman, G. L., Arch. Biochem. Biophys., 74:443-450 (1958). 0.1 ml of the material was mixed with 0.1 ml of Ellman reagent (i.e., 40 mg of 5,5'-dithiobis(2 nitrobenzoic) acid in 10 ml of pH 8.0 phosphate buffer). After 15 minutes of incubation, the absorbance was 412 nm. Cysteine was used as the standard sulfhydryl reagent.

Please replace the paragraph at page 17, lines 7-15, with the following amended paragraph:

The CRM₁₉₇ carrier protein was bromoacetylated according to the procedure described in Bernatowitz and Matsueda, Anal. Biochem., 155:95-102 (1986). Bromoacetic acid-N-hydroxy succinimide ester (available from Sigma Chemical Co.) in 100 mg/ml of dimethyl formamide was added dropwise to 3 ml of the protein (in 0.1M NaHCO₃) at a ratio of 1:1 (w/w) at 4°C. The solution was mixed and incubated for 1 hour at room temperature. The reaction mixture was then gel filtered on a Biegel Bio-Gel[®] P6 column as described above and the void fractions containing the bromoacetylated protein were pooled. Derivatization of amino groups on the carrier protein to the bromoacetyl groups was monitored by a decrease in the amount of free amino groups.

Please replace the paragraph at page 17, lines 17-21, with the following amended paragraph:

The bromoacetylated CRM₁₉₇ in 0.1 M NaHCO₃ was then added to the thiolated DeA-LPS at a 1:1.5 ratio of protein to LPS (w/w) in 0.1M NaHCO₃. The reaction mixture was incubated overnight at 4°C. The final conjugate (hereinafter referred to as "DeA-LPS-SPDP-CRM") was purified by gel filtration on a Biegel Bio-Gel[®] P30 (Bio-Rad) column equilibrated in 0.1 M NaHCO₃/1mM EDTA, pH7-9.

Please replace the paragraph at page 17, line 25 through page 18, line 3, with the following amended paragraph:

The immunogenicity of the DeA-LPS-SPDP-CRM conjugate prepared above was determined in Swiss Webster mice according to the following procedure. Groups of 6-8 week old female mice, 10 per group, were immunized subcutaneously with 10 µg LPS, 10 µg DeA-LPS, 10 µg DeA-LPS-SPDP (i.e., the unconjugated intermediate) and 10 µg of the DeA-LPS-SPDP-CRM conjugate. 10 µg CRM₁₉₇ was also administered to the mice to serve as a control. Each of these immunogens further contained 20 µg of ~~QS-21 (available from Aquila)~~ STIMULON™ QS-21 as an adjuvant in a final volume of 0.1 ml containing phosphate buffered saline (PBS), per dose. An additional group was immunized with 10 µg of LPS without the ~~QS24~~ STIMULON™ QS-21 adjuvant. The animals were immunized at weeks 0, 3, and 6 and blood samples were taken prior to each immunization for antibody determination. Blood samples were further taken at week 8 for antibody determination.

Please replace the paragraph at page 18, lines 12-21, with the following amended paragraph:

The purified LPS was diluted in endotoxin-free PBS to the following concentrations: 10 µg/mL for *Neisseria meningitidis* strains A1, H44/76, 2996 and Immunotypes L1, L2, L3, L4, L5, L6, L7, L8, L10, L11 and L12; and 2.5 µg/mL for the R6 strain. Polystyrene microtiter plates were coated with 100 µL per well of the diluted LPS-containing mixtures and incubated for 3 hours at 37°C followed by overnight storage at 4°C. The unbound LPS was then removed from the plates by suction utilizing an automatic plate washer. 150 µL per well of PBS/0.1% gelatin was then added to the plates and the plates were then incubated for 60 minutes at 37°C. Following this incubation, and between all subsequent steps, the plates

were washed with a mixture of PBS and 0.1% ~~Tween-20~~ Tween 20™ using an automatic plate washer.

Please replace the paragraph at page 18, lines 23-32, with the following amended paragraph:

Test mouse sera was serially diluted in a mixture of PBS, 0.05% ~~Tween-20~~ Tween 20™ and 0.1% gelatin. 100 µL per well of the dilution was added to the plates. The plates were incubated for 60 minutes at 37°C. Goat anti-mouse IgG alkaline phosphatase (from Southern Biotechnology), diluted in a mixture of PBS and 0.5% ~~Tween-20~~ Tween 20™, was then added in an amount of 100 µL per well and incubated for 60 minutes at 37°C. The color was developed using 100 µl of a 1mg/ml solution of p-nitrophenol phosphate in a diethanolamine buffer. These materials were allowed to react for 60 minutes at room temperature, after which the reaction was stopped by the addition of 50 µL per well of 3N NaOH. Absorbance values were determined using an automated ELISA reader with a 405 nm test and 690 nm reference filter.

Please replace the paragraph at page 20, line 8 through page 21, line 6, with the following amended paragraph:

The cross reactivity of anti-LPS conjugate antisera (the antisera against DeA-LPS-SPDP-CRM) was further examined by western blot analysis against purified LPS from various strains of several gram negative bacteria. Purified LPS samples of *Neisseria meningitidis*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, and *Helicobacter pylori* were first digested with protease and subjected to a standard SDS-PAGE (18%) separation procedure. The samples were then transferred to nitrocellulose membrane by standard western blot procedure. The membrane was blocked with 3% Bovine Serum Albumin (BSA) in

a mixture of PBS/0.05% ~~Tween-20~~ Tween 20™ for 30 min. and reacted with 1:100 dilution of test mouse sea. The blots were then washed with a mixture of PBS/0.05% ~~Tween-20~~ Tween 20™ and incubated with goat anti-mouse Ig alkaline phosphatase diluted in a mixture of PBS/0.05% Tween 20. Following the washing procedure, the blots were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium concentrate (NBT) phosphatase substrate system as described by the manufacturer (Kirkegaard and Perry laboratories, Inc., MD). The development procedure comprised mixing one part each of the BCIP and NBT concentrates with ten parts of Tris buffer solution in a glass container and adding these mixtures to the blots. After color development, the reaction was stopped by rinsing the blots with reagent quality water.